

Control of Fusarium Oxysporum Causing Fusarium Wilt by *Trichoderma Spp* and *Pseudomonas fluorescens* on *Arachis hypogaea L.*

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ABSTRACT:

Fusarium wilt diseases caused by the fungus *Fusarium oxysporum* lead to significant yield losses of crops. Biocontrol agents such as *Trichoderma viride*, *Trichoderma harzianum* and *Pseudomonas fluorescens* are applied to control Fusarium wilt of *Arachis hypogaea*. Experiments were conducted on the effect of culture filtrates of *T.viride* (1%), *T.harzianum* (1.5%), and *P. fluorescens* (2%) on biochemical parameters i.e aminonitrogen, nitratereductase, sucrose, starch, and proline. Plants sprayed with *T.viride* were found to have the highest aminonitrogen, nitratereductase, sucrose and starch content followed by those plants sprayed with *T.harzianum* and *P. fluorescens* compared to control plants. The proline content showed a sharp increase of about 2 times in the infected plants compared to that of control. The plants sprayed with *T.viride* showed slight increase in the proline content, followed by those sprayed with *T.harzianum* and *P. fluorescens*. This present study indicates that culture filtrate of *T.viride*(1%) is the most effective biocontrol agent in the inhibition of *Fusarium oxysporum* causing *Fusarium* wilt of *Arachis hypogaea L.*

Key words: *Fusarium oxysporum*, *Arachis hypogaea*. *Trichoderma viride*, *Trichoderma harzianum* and *Pseudomonas fluorescens*.

INTRODUCTION:

Arachis hypogaea L. (Groundnut) is one of the important crops all over the world. Especially in India, it is one of the major oil seed crops. In addition to this, being leguminous, groundnut has the ability to fix atmospheric nitrogen biologically into the soil which enriches the soil and this benefits the succeeding crop. The groundnut crop is equally vulnerable to soil borne

diseases as both the roots and pods of the plant grow in soil. Diseases caused by soil borne fungal pathogens reduce yields and the quality of the harvested pods. These diseases affect the crop plant until harvest. Pathogens attack all plant parts of groundnut and restrict plant development throughout the growing season as well as reducing seed quality in post-harvest storage[1].

Fusarium oxysporum, the soil borne pathogen causes vascular wilt diseases in a wide variety of economically important crops[2] . It has been known as most distributed and important fungal disease on field crops for many years. Vascular wilt has been a major limiting factor in the production of many agricultural and horticultural crops. The diseases are caused by different species of genus *Fusarium*. The antagonistic microorganisms act as biological control agents in resisting the pathogen. Baker and Cook defined biological control as the reduction of inoculum density or disease- producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host or antagonist, or by mass introduction of one or more antagonists.[3] This is an expanding field of research motivated by the hope that chemical control can, in part or even wholly, be replaced by biological and presumably more 'natural' methods that will do less harm to the environment. *Trichoderma* is a genus which include species of free-living soil fungi, opportunistic, avirulent plant symbionts[4], asymptomatic endophytes[5] and parasites of other fungi[6]. It is often the major component of the mycoflora in soils of various ecosystems, such as agricultural farm soil, grassland, forest, marshes, deserts and water[7,8,9]. *Trichoderma* species possess high reproductive capacity, ability to survive under very unfavourable conditions, efficiency in the utilization of nutrients, and capacity to modify the rhizosphere[10]. These fungi are well known for their ability to produce a wide range of antibiotic substances and for their ability to parasitize other fungi. These are highly interactive in roots, soil and foliar environments and have been used to control many crop pathogens. There have been studies on the application of antagonistic microbes, such as *Pseudomonas* spp., for control of *Fusarium* wilt [11,12,13]. Meena, Marimuthu, Vidyasekaran and Velazhahan

studied the biological control of root rot of groundnut with antagonistic *Pseudomonas fluorescens*. [14]

The leaves are the major sites of utilization of nitrates. The nitrates that are absorbed by plants are reduced to nitrites and then immediately to ammonia. Finally they are converted to amino acids and proteins. The nitrate reductase (NR) is the key enzyme in nitrogen metabolism, which converts the nitrate to nitrite. Sugars are precursors for the synthesis of phenolics, phytoalexins, lignin and callose. Hence, they play an important role in defense mechanism of plants against invading pathogens. In general, the infection by some pathogens brings changes in respiratory pathway and photosynthesis which are the vital processes taking place inside the plant leading to wide fluctuations in sugars [15] . Sugars play a major role in disease resistance by suppressing the pectinolytic and cellulolytic enzymes essential for pathogenesis [16]. The phenol and proline compounds act as adaptive mechanism in the host plant against the fungal infection. Proline accumulation is a common metabolic responses of higher plants to water deficits, and salinity stress, and has been the subject of numerous reviews over the last 20 years.[17,18,19]. Proline synthesis is implicated as a mechanism of alleviating cytoplasmic acidosis, and may maintain NADP⁺/NADPH ratios at values compatible with metabolism [20]. Rapid catabolism of proline upon relief of stress may provide reducing equivalents that support mitochondrial oxidative phosphorylation and the generation of ATP for recovery from stress and repair of stress-induced damage [21]. The present study deals with biocontrol activity of fusarium wilt of *Arachis hypogaea* treated with *Trichoderma viride*, *Trichoderma harzianum* and *Pseudomonas fluorescens* by focusing on the important biochemical parameters i.e. aminonitrogen, nitrate, reductase, sucrose, starch, and proline.

MATERIALS AND METHODS

Medium & Growth:

Fusarium oxysporum was grown on PSA for 30 days and further grown in Czapek's medium for 7 days and filtrate was taken. *Trichoderma viride* & *Trichoderma harzianum* were grown on Malt Extract agar and *Pseudomonas fluorescens* on ABM Medium and further grown on Czapek's medium in conical flask. It was further centrifuged and culture filtrate was taken

Four plants of *A. hypogaea* raised from the seeds (JLR –variety) were grown in each of six earthen pots (25cm diameter) upto 75 DAS and grouped into three sets.

Control-first set of two pots was sprayed with distilled water on 30 DAS and left without any treatment.

Infected- second set of two pots was sprayed with culture of pathogen, *Fusarium oxysporum* on 30 DAS and left without any treatment.

Infected-treated - third set was sprayed with pathogen on 30 DAS. These infected plants were sprayed with OIC of culture filtrates of antagonistic microorganisms, *Trichoderma viride* (1%), *Trichoderma harzianum* (1.5%) and *Pseudomonas fluorescens* on 40 DAS.

On 50 DAS, the leaves of control, infected and infected treated plants were collected for the estimation of biochemical parameters such as aminonitrogen, nitrate, reductase, sucrose, starch, and proline.

Preparation of Alcohol Extract

Fresh leaves were oven dried (80°C, 48h) and powdered using a mortar and pestle. 50 mg dried leaf powder was boiled in a water bath for 10 min with 10 ml of 80% ethyl alcohol. The homogenate was first cooled and then centrifuged at 600 rpm for 15 min. The supernatant was saved and made up to 20 ml with 80% ethyl alcohol. This extract was used for quantitative estimation of carbohydrates, phenols and nitrogen content. The residue was saved for starch estimation.

Amino Nitrogen

The pH of the alcoholic extract was adjusted to 7.0 by adding 0.1 N NaOH/HCl. To 1 ml of the above extract 1 ml of ninhydrin reagent was added. Then, it was heated for 20 min and cooled. 5 ml of distilled water was added and the absorbance was measured at 475nm in Systronics Spectrophotometer.

Nitrate Reductase Activity

Nitrate reductase activity was assayed by the method of [22] with suitable modifications [23]. Harvested fresh leaves were washed and cut into 5 mm bits. Leaf bits corresponding to 100 mg fresh weight were incubated in vials containing 5 ml of incubation medium. The incubation medium was prepared by mixing 0.1 N KNO₃ (1 ml), 0.1 M phosphate buffer of pH 7.5 (3.75 ml), 0.1% of Triton X-100 (0.01 ml) and 1% propanol (0.25 ml). Incubation was carried out in dark for one hour at room temperature (28± 2°C) giving occasional shakings. Aliquots of 0.2 ml from the incubation mixture were analysed for nitrite after 60 min. To 0.2 ml of incubation medium, 1.8 ml of distilled water, 1 ml of 3% sulphanilamide in 3 N HCl and 1 ml of 0.02% N-(1-naphthyl) ethylene-diamine dihydrochloride were added in quick succession. This was incubated for 15 min in darkness for colour development and absorbance was read at 540 nm with suitable blank in a Systronic Spectrophotometer. The amount of nitrite formed was expressed as nmoles of nitrite produced per minute per mg fresh weight using a sodium nitrite standard curve.

Sucrose

The sucrose content was estimated by the method of [24].

To 1 ml of the 80% ethanol extract 0.1 ml of 30% aqueous KOH was added and kept in a boiling water bath for 10 min. The samples were cooled and 3.0 ml of anthrone reagent was added and kept at 40°C for 10 min. The absorbance was

read at 620 nm. Glucose of known concentration was used as standard.

Starch

The starch content was estimated according to the method proposed by [25]

Extraction

The residue left behind after alcoholic extraction of the leaf materials was dissolved in 5 ml of 52% perchloric acid (PCA) for 1 h. The mixture was filtered through Whatman's filter paper (No. 42) and the filtrate was made up to 100 ml with distilled water.

To 1 ml of the PCA extract, 4 ml -of distilled water and 10 ml of freshly prepared cold anthrone reagent as added carefully along the side of the tube. The contents of the tubes were shaken vigorously and heated in a boiling water bath for 7.5 min. The tubes were then cooled immediately in running tap water and shaken well before reading the colour intensity at 630 nm in Systronics Spectrophotometer. The starch content was calculated with reference to glucose standard and multiplied by 0.9.

Proline

Extraction

The extraction and estimation of proline was done according to the method of [26]). 500 mg of fresh plant material was homogenized in a mortar and pestle with 10 ml of 3% aqueous sulfosalicylic acid. The homogenate was filtered through Whatman No. 2 filter paper and the residue was re-extracted. The extracts were

To 2.0 ml of the filtrate, 2.0 ml of acid ninhydrin and 2.0 ml of glacial acetic acid was added. The tubes were incubated for 1 h at 100°C on a water bath. The tubes were transferred on ice to terminate the reaction and 4.0 ml of toluene was added and mixed vigorously for 15-20 seconds. The chromophore containing toluene was aspirated from the aqueous phase. It was allowed to reach room temperature and the absorbance measured at 575 nm. A reagent blank was maintained. A standard curve was obtained using a known concentration of authentic proline. The proline content was expressed as mg of proline per gram fresh weight.

RESULTS

The highest amino nitrogen content was recorded in the control leaves (0.82 mg/g). The lowest amount of amino nitrogen was observed in the infected leaves (0.30 mg/g) which was 63% less than that of the control plants. Among the treatments the plants sprayed with *T.viride* were found to have the highest aminonitrogen content (0.61 mg/g), followed by those sprayed with *T.harzianum* (0.51 mg/g) and *P. fluorescens* (0.41 mg/g). The activity of NR enzyme was found to be highest in the control plants (1250 nmol/ mg). The least activity was recorded in the infected plants (900 nmol/ mg). Among the treatments , maximum activity was recorded in the leaves sprayed with *T.viride* (1575 nmol/ mg) , followed by those sprayed with *T.harzianum* (1423 nmol/ mg) and *P. fluorescens* (1350 nmol/ mg). (Table1).

Treatments	Amino nitrogen (mg/g.d.w)	Nitrate reductase activity (nmolNO ₂ /mg/m)
Control plants	0.82 ± 0.13	1250 ± 0.57
Plants infected with <i>F.oxysporum</i>	0.30 ± 0.11 ^b	900 ± 1.00 ^a
Infected plants treated with <i>T.viride</i>	0.61 ± 0.12	1575 ± 0.57 ^a
Infected plants treated with <i>T. harzianum</i>	0.51 ± 0.11	1423 ± 1.00 ^a
Infected plants treated with <i>P. fluorescens</i> .	0.41 ± 0.12 ^a	1350 ± 1.00 ^a

pooled and made up to 20 ml with aqueous sulfosalicylic acid and used for the estimation.

Table1. Effect of culture filtrates of *T.viride*, *T.harzianum* and *P. fluorescens* on aminonitrogen

content and nitrate reductase activity of *Arachis hypogaea* leaves infected with *F.oxysporum*
^ap< 0.05 , ^bp<0.01 as compared to control

^ap< 0.001 as compared to control

The leaves of control plants recorded highest sucrose content (8.44mg/g) and the lowest sucrose content was recorded in the infected leaves, (3.12 mg/g). Among the treatments the plants sprayed with *T.viride* were found to show the highest sucrose content (6.64 mg/g), followed by those sprayed with *T.harzianum* (5.44 mg/g) and *P. fluorescens* (4.82 mg/g). The leaves of control plants recorded highest starch content (51.89 mg/g) and the lowest starch content was recorded in the infected leaves, (21.29 mg/g) which was (58.97%) less than that of the control ones. Among the treatments the plants sprayed with *T.viride* were found to show the highest starch content (42.65 mg/g), followed by those sprayed with *T.harzianum* (37.24 mg/g) and *P. fluorescens* (32.94 mg/g).(Table2).

Treatments	Sucrose (mg/g.f.w)	Starch (mg/g.f.w)
Control plants	8.44 ±0.18	51.89±0.43
Plants infected with <i>F.oxysporum</i>	3.12 ± 0.10 ^a	21.29± 0.11 ^a
Infected plants treated with <i>T.viride</i>	6.64 ± 0.15 ^a	42.65 ±0.27 ^a
Infected plants treated with <i>T. harzianum</i>	5.44 ±0.14 ^a	37.24 ±0.16 ^a
Infected plants treated with <i>P. fluorescens</i> .	4.82 ±0.12 ^a	32.94±0.12 ^a

Table 2. Effect of culture filtrates of *T.viride*, *T.harzianum* and *P. fluorescens* on sucrose and starch content of *Arachis hypogaea* leaves infected with *F.oxysporum*

^ap< 0.001 as compared to control

The values within a column followed by different letters are significantly different according to Tukey's HSD multiple range test (TMRT) at 5% level of significance (n=3)

The proline content showed a sharp increase of about 2 times (4.56 mg/g) in the infected plants compared to that of control (2.01 mg/g) plants. Among the treatments, the plants sprayed with *T.viride* showed slight increase in the proline content (2.38 mg/g), followed by those sprayed with *T.harzianum* (2.60 mg/g) and *P. fluorescens* (2.79 mg/g).(Fig1)

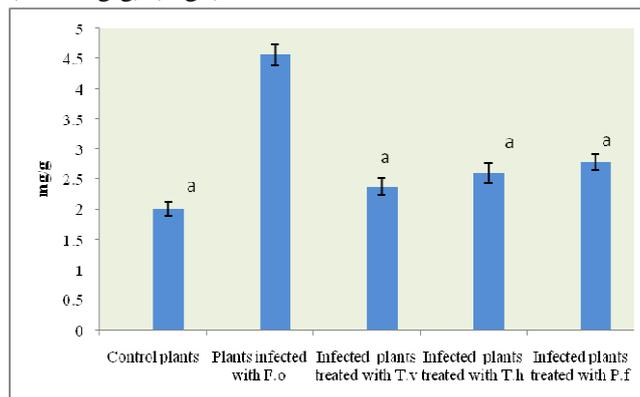


Fig1 Effect of culture filtrates of *T.viride*, *T.harzianum* and *P. fluorescens* on proline content (mg/g.f.w) of *Arachis hypogaea* leaves infected with *F.oxysporum*

^ap< 0.001 as compared to control

The values within a column followed by different letters are significantly different according to Tukey's HSD multiple range test (TMRT) at 5% level of significance (n=3)

DISCUSSION

Significant reduction in amino nitrogen and NR activity was observed in the *Fusarium oxysporum* - infected plants. Similar observation was made by Prasad that the heavy reduction in nitrogen content in banana plant under fungal pathogenesis was due to the fact that the pathogen survived at the expense of host nitrogen pool leading to the enhancement of disease development, which brought down the protein and total nitrogen content in the host tissue. [27] According to Padma Singh decreased nitrogen content in the *Alternaria sp.* -infected onion leaves was due to the disruption of cell structure coupled with enhanced proteolytic enzyme

activity which enhanced disease development[28].Of all the treatments, *T.viride* treatment showed highest NR activity followed by *T.harzianum*-sprayed plants and *P.fluorescens*-sprayed plants compared to control plants.The *P. fluorescens*-sprayed leaves recorded least reduction in amino nitrogen and NR activity over other treatments. This might be due to the fact that the treatment may induce the activity of the enzymes involved in the nitrogen metabolism. The least reduction might be due to their participation of defense reaction against the pathogen infection [29,30]. John, Rojan, Tyagi, and Prévost, observed that soybean plants infected with *Pythium arrhenomanes* and *Fusarium oxysporum f.sp.adzuki* pathogens treated with *Trichoderma viride* showed higher total nitrogen, carbon and dry weight and established the positive effect of the biocontrol agent on enhancement of plant growth.[31]

The sucrose and starch was found to be drastically reduced in the infected plant. The pathogen in the infected tissue normally uses the host carbohydrate metabolites for their growth and survival. This is in accordance with the work of Chakrabarty, Mukewar, Rai and Saravan in cotton plant infected with grey mildew disease[32].Debnath, Sharma and Kant reported that the decreased starch content in the *Brassica* leaves infected with *Albugo* sp. was mainly due to the fact that these substances were utilized by the pathogen for its growth and development.[33].Least reduction of starch and starch content in *T. viride*-sprayed plants was followed by *T.harzianum*-sprayed plants and *P.fluorescens*- sprayed plants .The least reduction in the bioagents-sprayed leaves may be due to induction of the treated plants (SR) to utilize the carbohydrates for the biosynthesis of phenolic compounds. These phenolic compounds were used for the defense reaction against the pathogen infection. This finding substantiates the works of Neish and Rajavel in *Pseudomonas*- and *Trichoderma*-treated *Capsicum* fruits infected

with *C. capsici*.[34,35] The phenol and proline compounds act as adaptive mechanism in the host plant against the fungal infection. Accumulation of phenolic compounds and proline at the infection site has been correlated with the restriction of pathogen development, since such compounds are toxic to pathogens. Proline protects membranes and proteins against the adverse effects of high concentrations of inorganic ions and temperature extremes [36,37,38] . Proline may also function as a protein-compatible hydrotrope. [39].Of all the treatments *T. viride* - sprayed plants (1%) showed a minimum increase in the proline content followed by *T.harzianum*-sprayed plants and *P.fluorescens*- sprayed plants. Proline protects membranes as a hydroxyl radical scavenger.[40] The treatments generally induced systemic resistance in the host cell, which in turn enhanced activation of these enzymes in the conversion of reactive oxygen species or radicals to water in order to reduce the infection. Thus the rapid conversion reduced the severity of the infection caused by the pathogen.

CONCLUSION

Among the treatments the plants sprayed with *T.viride* were found to have the highest aminonitrogen, nitratereductase, sucrose starch content followed by those sprayed with *T.harzianum* and *P. fluorescens*. The plants sprayed with *T.viride* showed slight increase in the proline content ,followed by those sprayed with *T.harzianum* and *P. fluorescens* All these biocontrol agents (*T. viride* , *T.harzianum* and *P. fluorescens*) are effective in the control of Fusarium wilt of groundnut with slight variations in each biochemical parameters. *Trichoderma* spp. as bio-control agents induced the accumulation of some enzymes chitinase, peroxidase and polyphenol oxidase which played an important role in plant defense mechanisms against pathogen infection and significantly reduced the disease.[41]Of all the treatments, *T.*

viride treatment showed higher rate of inhibition of Pectinolytic and cellulolytic enzymes of *Fusarium oxysporum* followed by that of *T.harzianum* and *P.fluorescens*. [42] This study concludes that culture filtrate of *T.viride*(1%) is the most effective biocontrol agent in the inhibition of *Fusarium oxysporum* causing *Fusarium* wilt of *Arachis hypogaea* .L

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